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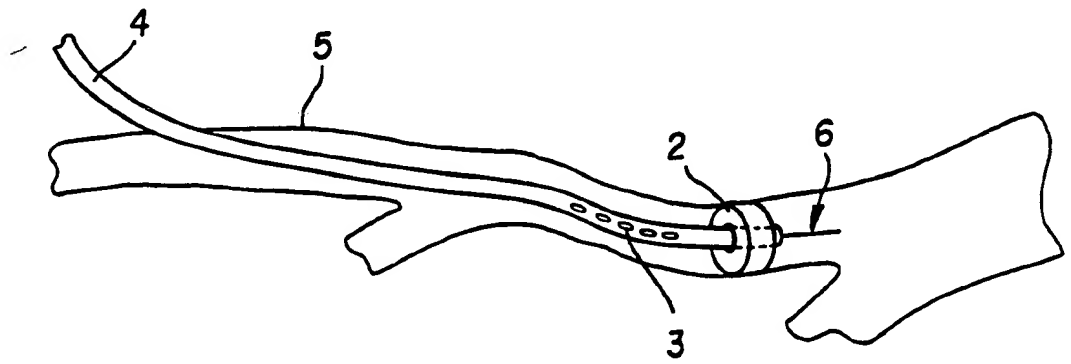
(54) Title: TREATMENT OF DISEASES BY SITE-SPECIFIC INSTILLATION OF CELLS OR SITE-SPECIFIC TRANSFORMATION OF CELLS AND KITS THEREFOR



(57) Abstract

A method for the direct treatment towards the specific sites of a disease is disclosed. The method is based on the delivery of proteins by catheterization to discrete blood vessel segments using genetically modified or normal cells or other vector systems. Endothelial cells expressing recombinant therapeutic agent or diagnostic proteins are situated on the walls (5) of the blood vessel or in the tissue perfused by the vessel in a patient. This technique provides for the transfer of cells or vectors and expression of recombinant genes *in vivo* and allows the introduction of proteins of therapeutic or diagnostic value for the treatment of diseases.

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Description

Treatment of Diseases By Site-Specific Instillation Of Cells Or Site-Specific Transformation Of Cells And Kits Therefor

Technical Field:

The present invention relates to the treatment of diseases by the site-specific instillation or transformation of cells and kits therefor.

Background Art:

The effective treatment of many systemic and inherited diseases remains a major challenge to modern medicine. The ability to deliver therapeutic agents to specific sites in vivo would be an asset in the treatment of, e.g., localized diseases. In addition the ability to cause a therapeutic agent to perfuse through the circulatory system would be effective for the treatment of, e.g., systemic diseases.

For example, it would be desirable to administer in a steady fashion an antitumor agent or toxin in close proximity to a tumor. Similarly, it would be desirable to cause a perfusion of, e.g., insulin in the blood of a person suffering from diabetes. However, for many therapeutic agents there is no satisfactory method of either site-specific or systemic administration.

In addition, for many diseases, it would be desirable to cause, either locally or systemically, the expression of a defective endogenous gene, the expression of a exogenous gene, or the suppression of an endogenous gene. Again, these remain unrealized goals.

In particular, the pathogenesis of atherosclerosis is

characterized by three fundamental biological processes. These are: 1) proliferation of intimal smooth muscle cells together with accumulated macrophages; 2) formation by the proliferated smooth muscle cells of large amounts of connective tissue matrix; and 3) accumulation of lipid, principally in the form of cholesterol esters and free cholesterol, within cells as well as in surrounding-connective tissue.

Endothelial cell injury is an initiating event and is manifested by interference with the permeability barrier of the endothelium, alterations in the non-thrombogenic properties of the endothelial surface, and promotion of procoagulant properties of the endothelium. Monocytes migrate between endothelial cells, become active as scavenger cells, and differentiate into macrophages.

Macrophages then synthesize and secrete growth factors including platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor alpha (TGF-α). These growth factors are extremely potent in stimulating the migration and proliferation of fibroblasts and smooth muscle cells in the atherosclerotic plaque. In addition, platelets may interact with the injured endothelial cell and the activated macrophage to potentiate the elaboration of growth factors and thrombus formation.

Two major problems in the clinical management of coronary artery disease include thrombus formation in acute myocardial ischemia and restenosis following coronary angioplasty (PTCA). Both involve common cellular events, including endothelial injury and release of potent growth factors by activated macrophages and platelets. Coronary angioplasty produces

fracturing of the atherosclerotic plaque and removal of the endothelium. This vascular trauma promotes platelet aggregation and thrombus formation at the PTCA site. Further release of mitogens from platelets and macrophages, smooth muscle cell proliferation and monocyte infiltration result in restenosis.

Empiric therapy with antiplatelet drugs has not prevented this problem, which occurs in one-third of patients undergoing PTCA. A solution to restenosis is to prevent platelet aggregation, thrombus formation, and smooth muscle cell proliferation.

Thrombus formation is also a critical cellular event in the transition from stable to unstable coronary syndromes. The pathogenesis most likely involves acute endothelial cell injury and or plaque rupture, promoting dysjunction of endothelial cell attachment, and leading to the exposure of underlying macrophage foam cells. This permits the opportunity for circulating platelets to adhere, aggregate, and form thrombi.

The intravenous administration of thrombolytic agents, such as tissue plasminogen activator (tPA) results in lysis of thrombus in approximately 70% of patients experiencing an acute myocardial infarction. Nonetheless, approximately 30% of patients fail to reperfuse, and of those patients who undergo initial reperfusion of the infarct related artery, approximately 25% experience recurrent thrombosis within 24 hours. Therefore, an effective therapy for rethrombosis remains a major therapeutic challenge facing the medical community today.

As noted above, an effective therapy for rethrombosis is

by far not the only major therapeutic challenge existing today. Others include the treatment of other ischemic conditions, including unstable angina, myocardial infarction or chronic tissue ischemia, or even the treatment of systemic and inherited diseases or cancers. These might be treated by the effective administration of anticoagulants, vasodilatory, angiogenic, growth factors or growth inhibitors to a patient. Thus, there remains a strongly felt need for an effective therapy in all of these clinical settings.

Disclosure of the Invention

Accordingly, one object of the present invention is to provide a novel method for the site-specific administration of a therapeutic agent.

It is another object of the present invention to provide a method for the perfusion of a therapeutic agent in the blood stream of a patient.

It is another object of the present invention to provide a method for causing the expression of an exogenous gene in a patient.

It is another object of the present invention to provide a method for causing the expression of a defective endogenous gene in a patient.

It is another object of the present invention to provide a method for suppressing the expression of an endogenous gene in a patient.

It is another object of the present invention to

provide a method for site-specifically replacing damaged cells in a patient.

It is another object of the present invention to provide a method for the treatment of a disease by causing either the site-specific administration of a therapeutic agent or the perfusion of a therapeutic agent in the bloodstream of a patient.

It is another object of the present invention to provide a method for the treatment of a disease by causing either the expression of an exogenous gene, the expression of a defective endogenous gene, or the suppression of the expression of an endogenous gene in a patient.

It is another object of the present invention to provide a method for the treatment of a disease by site-specifically replacing damaged cells in a patient.

It is another object of the present invention to provide a kit for site-specifically instilling normal or transformed cells in a patient.

It is another object of the present invention to provide a kit for site-specifically transforming cells in vivo.

These and other objects of this invention which will become apparent during the course of the following detailed description of the invention have been discovered by the inventors to be achieved by (a) a method which comprises either (i) site-specific instillation or either normal (untransformed) or transformed cells in a patient or (ii) site-specific transformation of cells in a patient and (b) a kit which

contains a catheter for (i) site-specific instillation of either normal or transformed cells or (ii) site-specific transformation of cells.

Site-specific instillation of normal cells can be used to replace damaged cells, while instillation of transformed cells can be used to cause the expression of either a defective endogenous gene or an exogenous gene or the suppression of an endogenous gene product. Instillation of cells in the walls of the patient's blood vessels can be used to cause the steady perfusion of a therapeutic agent in the blood stream.

Brief Description of the Drawings

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying figures, wherein:

FIGURES 1 and 2 illustrate the use of a catheter in accordance with the invention to surgically or percutaneously implant cells in a blood vessel or to transform in vivo cells present on the wall of a patient's blood vessel.

Best Mode For Carrying Out the Invention

Thus, in one embodiment, the present invention is used to treat diseases, such as inherited diseases, systemic diseases, diseases of the cardiovascular system, diseases of particular organs, or tumors by instilling normal or transformed cells or by transforming cells.

The cells which may be instilled in the present method include endothelium, smooth muscle, fibroblasts, monocytes, macrophages, and parenchymal cells. These cells may produce proteins which may have a therapeutic or diagnostic effect and which may be naturally occurring or arise from recombinant genetic material.

Referring now to the figures, wherein like reference numerals designate identical or corresponding parts throughout the several views, and more particularly to FIGURE 1 thereof, this figure illustrates the practice of the present invention with a catheter having a design as disclosed in U.S. Patent 4,636,195, which is hereby incorporated by reference. This catheter may be used to provide normal or genetically altered cells on the walls of a vessel or to introduce vectors for the local transformation of cells. In the figure, 5 is the wall of the blood vessel. The figure shows the catheter body 4 held in place by the inflation of inflatable balloon means 1 and 2. The section of the catheter body 4 situated between balloon means 1 and 2 is equipped with instillation port means 3. The catheter may be further equipped with a guidewire means 6. FIGURE 2 illustrates the use of a similar catheter, distinguished from the catheter illustrated in Figure 1 by the fact that it is equipped with only a single inflatable balloon means 2 and a plurality of instillation port means 3. This catheter may contain up to twelve individual instillation port means 3, with five being illustrated.

In the case of delivery to an organ, the catheter may be introduced into the major artery supplying the tissue. Cells containing recombinant genes or vectors can be introduced through a central instillation port after temporary occlusion of the arterial circulation. In this way, cells or vector DNA

may be delivered to a large amount of parenchymal tissue distributed through the capillary circulation. Recombinant genes can also be introduced into the vasculature using the double balloon catheter technique in the arterial circulation proximal to the target organ. In this way, the recombinant genes may be secreted directly into the circulation which perfuse the involved tissue or may be synthesized directly within the organ.

In one embodiment, the therapeutic agents are secreted by vascular cells supplying specific organs affected by the disease. For example, ischemic cardiomyopathy may be treated by introducing angiogenic factors into the coronary circulation. This approach may also be used for peripheral vascular or cerebrovascular diseases where angiogenic factors may improve circulation to the brain or other tissues. Diabetes mellitus may be treated by introduction of glucose-responsive insulin secreting cells in the portal circulation where the liver normally sees a higher insulin concentration than other tissues.

In addition to providing local concentrations of therapeutic agents, the present method may also be used for delivery of recombinant genes to parenchymal tissues, because high concentrations of viral vector and other vectors can be delivered to a specific circulation. Using this approach, deficiencies of organ-specific proteins may also be treated. For example, in the liver, α-antitrypsin inhibitor deficiency or hyperchloresterolemia may be treated by introduction of α-antitrypsin or the LDL receptor gene. In addition, this approach may be used for the treatment of malignancy. Secretion of specific recombinant toxin genes into the circulation of inoperable-tumors provides a therapeutic effect.

Examples include acoustic neuromas or certain hemangiomas which are otherwise unresectable.

In clinical settings, these therapeutic recombinant genes are introduced in cells supplying the circulation of the involved organ. Although the arterial and capillary circulations are the preferred locations for introduction of these cells, venous systems are also suitable.

In its application to the treatment of local vascular damage the present invention provides for the expression of proteins which ameliorate this condition in situ. In one embodiment, because vascular cells are found at these sites, they are used as carriers to convey the therapeutic agents.

The invention thus, in one of its aspects, relies on genetic alteration of endothelial and other vascular cells or somatic cell gene therapy, for transmitting therapeutic agents (i.e., proteins, growth factors) to the localized region of vessel injury. To successfully use gene transplantation in the cells, four requirements must be fulfilled. First, the gene which is to be implanted into the cell must be identified and isolated. Second, the gene to be expressed must be cloned and available for genetic manipulation. Third, the gene must be introduced into the cell in a form that will be expressed or functional. Fourth, the genetically altered cells must be situated in the vascular region where it is needed.

In accordance with the present invention the altered cells or appropriate vector may be surgically, percutaneously, or intravenously introduced and attached to a section of a patient's vessel wall. Alternatively, some of the cells existing on the patient's vessel wall are transformed with the

desired genetic material or by directly applying the vector. In some instances, vascular cells which are not genetically modified can be introduced by these methods to replace cells lost or damaged on the vessel surface.

Any blood vessel may be treated in accordance with this invention; that is, arteries, veins, and capillaries. These blood vessels may be in or near any organ in the human, or mammalian, body.

Introduction of normal or genetically altered cells into a blood vessel:

This embodiment of the invention may be illustrated as follows:

I. Establishment of endothelial or other vascular cells in tissue culture.

Initially, a cell line is established and stored in liquid nitrogen. Prior to cryopreservation, an aliquot is taken for infection or transfection with a vector, viral or otherwise, containing the desired genetic material.

Endothelial or other vascular cells may be derived enzymatically from a segment of a blood vessel, using techniques previously described in J.W. Ford, et al., In Vitro, 17, 40 (1981). The vessel is excised, inverted over a stainless steel rod and incubated in 0.1% trypsin in Ca^{++}- and Mg^{++}- free Hank's balanced salt solution (BSS) with 0.125% EDTA at pH 8 for 10 min at 37°C.

Cells (0.4 to 1.5 x 10^6) are collected by centrifugation

and resuspended in medium 199 (GIBCO) containing 10% fetal bovine serum, endothelial cell growth supplement (ECGS, Collaborative Research, Waltham, MA) at 25 μg/ml, heparin at 15 U/ml, and gentamicin (50 μg/ml). Cells are added to a 75 cm^2 tissue culture flask precoated with gelatin (2 mg/ml in distilled water). Cells are fed every second day in the above medium until they reach confluence.

After two weeks in culture, the ECGS and heparin may be omitted from the medium when culturing porcine endothelium. If vascular smooth muscle cells or fibroblaits are desired the heparin and ECGS can be omitted entirely from the culturing procedure. Aliquots of cells are stored in liquid nitrogen by resuspending to approximately 10^6 cells in 0.5 ml of ice cold fetal calf serum on ice. An equal volume of ice cold fetal calf serum containing 10% DMSO is added, and cells are transferred to a prechilled screw cap Corning freezing tube. These cells are transferred to a -70°C freezer for 3 hours before long term storage in liquid nitrogen.

The cells are then infected with a vector containing the desired genetic material.

II. Introduction of cells expressing normal or exogenous proteins into the vasculature.

A. Introduction of cells expressing relevant proteins by catheterization.

The patient is prepared for catheterization either by surgery or percutaneously, observing strict adherence to sterile techniques. A cutdown procedure is performed over the target blood vessel or a needle is inserted into the target

blood vessel after appropriate anesthesia. The vessel (5) is punctured and a catheter, such as described in U.S. Patent 4,636,195, which is hereby incorporated by reference (available from USCI, Billerica, MA) is advanced by guidewire means (6) under fluoroscopic guidance, if necessary, into the vessel (5) (Figure 1). This catheter means (4) is designed to introduce infected endothelial cells into a discrete region of the artery. The catheter has a proximal and distal balloon means (2) and (1), respectively, (e.g., each balloon means may be about 3 mm in length and about 4 mm in width), with a length of catheter means between the balloons. The length of catheter means between the balloons has a port means connected to an instillation port means (3). When the proximal and distal balloons are inflated, a central space is created in the vessel, allowing for instillation of infected cells though the port.

A region of the blood vessel is identified by anatomical landmarks and the proximal balloon means (2) is inflated to denude the endothelium by mechanical trauma (e.g., by forceful passage of a partially inflated balloon catheter within the vessel) or by mechanical trauma in combination with small amounts of a proteolytic enzyme such as dispase, trypsin, collagenase, papain, pepsin, chymotrypsin or cathepsin, or by incubation with these proteolytic enzymes alone. In addition to proteolytic enzymes, lipases may be used. The region of the blood vessel may also be denuded by treatment with a mild detergent or the like, such as NP-40, Triton X100, deoxycholate, or SDS.

The denudation conditions are adjusted to achieve essentially complete loss of endothelium for cell transfers or approximately 20 to 90%, preferably 50 to 75%, loss of cells

from the vessel wall for direct infection. In some instances cell removal may not be necessary. The catheter is then advanced so that the instillation port means (3) is placed in the region of denuded endothelium. Infected, transfected or normal cells are then instilled into the discrete section of artery over thirty minutes. If the blood vessel is perfusing an organ which can tolerate some ischemia, e.g., skeletal muscle, distal perfusion is not a major problem, but can be restored by an external shunt if necessary, or by using a catheter which allows distal perfusion. After instillation of the infected endothelial cells, the balloon catheter is removed, and the arterial puncture site and local skin incision are repaired. If distal perfusion is necessary, an alternative catheter designed to allow distal perfusion may be used.

B. Introduction of recombinant genes directly into cells on the wall of a blood vessel or perfused by a specific circulation in vivo; infection or transfection of cells on the vessel wall and organs.

Surgical techniques are used as described above. Instead of using infected cells, a high titer desired genetic material transducing viral vector (10^5 to 10^6 particles/ml) or DNA complexed to a delivery vector is directly instilled into the vessel wall using the double balloon catheter technique. This vector is instilled in medium containing serum and polybrene (10 μg/ml) to enhance the efficiency of infection. After incubation in the dead space created by the catheter for an adequate period of time (0.2 to 2 hours or greater), this medium is evacuated, gently washed with phosphate-buffered saline, and arterial circulation is restored. Similar protocols are used for post operative recovery.

The vessel surface can be prepared by mechanical denudation alone, in combination with small amounts of proteolytic enzymes such as dispase, trypsin, collagenase or cathepsin, or by incubation with these proteolytic enzymes alone. The denudation conditions are adjusted to achieve the appropriate loss of cells from the vessel wall.

Viral vector or DNA-vector complex is instilled in Dulbecco 's modified Eagle's medium using purified virus or complexes containing autologous serum, and adhesive molecules such as polybrene (10 μg/ml), poly-L-lysine, dextran sulfate, or any polycationic substance which is physiologically suitable, or a hybrid antibody directed against the envelope glycoprotein of the virus or the vector and the relevant target in the vessel wall or in the tissue-perfused by the vessel to enhance the efficiency of infection by increasing adhesion of viral particles to the relevant target cells. The hybrid antibody directed against the envelope glycoprotein of the virus or the vector and the relevant target cell can be made by one of two methods. Antibodies directed against different epitopes can be chemically crosslinked (G. Jung, C.J. Honsik, R.A. Reisfeld, and H.J. Muller-Eberhard, Proc. Natl. Acad. Sci. USA, 83, 4479 (1986); U.D. Staerz, O. Kanagawa, and M.J. Bevan, Nature, 314, 628 (1985); and P. Perez, R.W. Hoffman, J.A. Titus, and D.M. Segal, J. Exp. Med., 163, 166 (1986)) or biologically coupled using hybrid hybridomas (U.D. Staerz and M.J. Bevan, Proc. Natl. Acad. Sci. USA, 83, 1453 (1986); and C. Milstein and A.C. Cuello, Nature, 305, 537 (1983)). After incubation in the central space of the catheter for 0.2 to 2 hours or more, the medium is evacuated, gently washed with phosphate buffered saline, and circulation restored.

Using a different catheter design (See Figure 2), a

different protocol for instillation can also be used. This second approach involves the use of a single balloon means (2) catheter with multiple port means (3) which allow for high pressure delivery of the retrovirus into partially denuded arterial segments. The vessel surface is prepared as described above and defective vector is introduced using similar adhesive molecules. In this instance, the use of a high pressure delivery system serves to optimize the interaction of vectors with cells in adjacent vascular tissue.

The present invention also provides for the use of growth factors delivered locally by catheter or systemically to enhance the efficiency of infection. In addition to retroviral vectors, herpes virus, adenovirus, or other viral vectors are suitable vectors for the present technique.

It is also possible to transform cells within an organ or tissue. Direct transformation of organ or tissue cells may be accomplished by one of two methods. In a first method a high pressure transfection is used. The high pressure will cause the vector to migrate through the blood vessel walls into the surrounding tissue. In a second method, injection into a capillary bed, optionally after injury to allow leaking, gives rise to direct infection of the surrounding tissues.

The time required for the instillation of the vectors or cells will depend on the particular aspect of the invention being employed. Thus, for instilling cells or, vectors in a blood vessel a suitable time would be from 0.01 to 12 hrs, preferably 0.1 to 6 hrs, most preferably 0.2 to 2 hrs. Alternatively for high pressure instillation of vectors or cells, shorter times might be preferred.

Obtaining the cells used in this invention:

The term "genetic material" generally refers to DNA which codes for a protein. This phrase also encompasses RNA when used with an RNA virus or other vector based on RNA.

Transformation is the process by which cells have incorporated an exogenous gene by direct infection, transfection or other means of uptake.

The term "vector" is well understood and is synonymous with the often-used phrase "cloning vehicle". A vector is non-chromosomal double-stranded DNA comprising an intact replicon such that the vector is replicated when placed within a unicellular organism, for example by a process of transformation. Viral vectors include retroviruses, adenoviruses, herpesvirus, papovirus, or otherwise modified naturally occurring viruses. Vector also means a formulation of DNA with a chemical or substance which allows uptake by cells.

In another embodiment the present invention provides for inhibiting the expression of a gene. Four approaches may be utilized to accomplish this goal. These include the use of antisense agents, either synthetic oligonucleotides which are complementary to the mRNA (Maher III, L.J. and Dolnick, B.J. Arch. Biochem. Biophys., 253, 214-220 (1987) and (Zamecnik, P.C., et al., Proc. Natl. Acad. Sci., 83, 4143-4146 (1986)), or the use of plasmids expressing the reverse complement of this gene (Izant, J.H. and Weintraub, H., Science, 229, 345-352, (1985); Cell, 36, 1077-1015 (1984)). In addition, catalytic RNAS, called ribozymes, can specifically degrade RNA sequences (Uhlenbeck, O.C., Nature, 328, 596-600 (1987), Haseloff, J. and

Gerlach, W.L., Nature, 334, 585-591 (1988)). The third approach involves "intracellular immunization", where analogues of intracellular proteins can interfere specifically with their function (Friedman, A.D., Triezenberg, S.J. and McKnight, S.L., Nature, 335, 452-454 (1988)), described in detail below.

The first approaches may be used to specifically eliminate transcripts in cells. The loss of transcript may be confirmed by S1 nuclease analysis, and expression of binding protein determined using a functional assay. Single-stranded oligonucleotide analogues may be used to interfere with the processing or translation of the transcription factor mRNA. Briefly, synthetic oligonucleotides or thiol-derivative analogues (20-50 nucleotides) complementary to the coding strand of the target gene may be prepared. These antisense agents may be prepared against different regions of the MRNA. They are complementary to the 5' untranslated region, the translational initiation site and subsequent 20-50 base pairs, the central coding region, or the 3' untranslated region of the gene. The antisense agents may be incubated with cells transfected prior to activation. The efficacy of antisense competitors directed at different portions of the messenger RNA may be compared to determine whether specific regions may be more effective in preventing the expression of these genes.

RNA can also function in an autocatalytic fashion to cause autolysis or to specifically degrade complementary RNA sequences (Uhlenbeck, O.C., Nature, 328, 596-600 (1987), Haseloff, J. and Gerlach, W.L., Nature, 334, 585-591 (1988), and Hutchins, C.J., et al, Nucleic Acids Res., 14, 3627-3640 (1986)). The requirements for a successful RNA cleavage include a hammerhead structure with conserved RNA sequence at the region flanking this structure. Regions adjacent to this

catalytic domain are made complementary to a specific RNA, thus targeting the ribozyme to specific cellular mRNAs. To inhibit the production of a specific target gene, the mRNA encoding this gene may be specifically degraded using ribozymes. Briefly, any GUG sequence within the RNA transcript can serve as a target for degradation by the ribozyme. These may be identified by DNA sequence analysis and GUG sites spanning th RNA transcript may be used for specific degradation. Sites in the 5' untranslated region, in the coding region, and in the 3' untranslated region may be targeted to determine whether one region is more efficient in degrading this transcript. Synthetic oligonucleotides encoding 20 base pairs of complementary sequence upstream of the GUG site, the hammerhead structure and ~20 base pairs of complementary sequence downstream of this site may be inserted at the relevant site in the cDNA. In this way, the ribozyme may be targeted to the same cellular compartment as the endogenous message. The ribozymes inserted downstream of specific enhancers, which give high level expression in specific cells may also be generated. These plasmids may be introduced into relevant target cells using electroporation and cotransfection with a neomycin resistant plasmid, pSV2-Neo or another selectable marker. The expression of these transcripts may be confirmed by Northern blot and S1 nuclease analysis. When confirmed, the expression of mRNA may be evaluated by S1 nuclease protection to determine whether expression of these transcripts reduces steady state levels of the target MRNA and the genes which it regulates. The level of protein may also be examined.

Genes may also be inhibited by preparing mutant transcripts lacking domains required for activation. Briefly, after the domain has been identified, a mutant form which is incapable f stimulating function is synthesized. This

truncated gene product may be inserted downstream of the SV-40 enhancer in a plasmid containing the neomycin resistance gene (Mulligan, R. and Berg, P., Science, 209, 1422-1427 (1980) (in a separate transcription unit). This plasmid may be introduced into cells and selected using G418. The presence of the mutant form of this gene will be confirmed by S1 nuclease analysis and by immunoprecipitation. The function of the endogenous protein in these cells may be evaluated in two ways. First, the expression of the normal gene may be examined. Second, the known function of these proteins may be evaluated. In the event that this mutant intercellular interfering form is toxic to its host cell, it may be introduced on an inducible control element, such as metallothionein promoter. After the isolation of stable lines, cells may be incubated with Zn or Cd to express this gene. Its effect on host cells can then be evaluated.

Another approach to the inactivation of specific genes is to overexpress recombinant proteins which antagonize the expression or function of other activities. For example, if one wished to decrease expression of TPA (e.g., in a clinical setting of disseminate thrombolysis), one could overexpress plasminogen activator inhibitor.

Advances in biochemistry and molecular biology in recent years have led to the construction of "recombinant" vectors in which, for example, retroviruses and plasmids are made to contain exogenous RNA or DNA, respectively. In particular instances the recombinant vector can include heterologous RNA or DNA, by which is meant RNA or DNA that codes for a polypeptide ordinarily not produced by the organism susceptible to transformation by the recombinant vector. The production of recombinant RNA and DNA vectors is well understood and need not

be described in detail. However, a brief description of this process is included here for reference.

For example, a retrovirus or a plasmid vector can be cleaved to provide linear RNA or DNA having ligatable termini. These termini are bound to exogenous RNA or DNA having complementary like ligatable termini to provide a biologically functional recombinant RNA or DNA molecule having an intact replicon and a desired phenotypical property.

A variety of techniques are available for RNA and DNA recombination in which adjoining ends of separate RNA or DNA fragments are tailored to facilitate ligation.

The exogenous, i.e., donor, RNA or DNA used in the present invention is obtained from suitable cells. The vector is constructed using known techniques to obtain a transformed cell capable of in vivo expression of the therapeutic agent protein. The transformed cell is obtained by contacting a target cell with a RNA or DNA containing formulation permitting transfer and uptake of the RNA or DNA into the target cell. Such formulations include, for example, retroviruses, plasmids, liposomal formulations, or plasmids complexes with polycationic substances such as poly-L-lysine, DEAC-dextran and targeting ligands.

The present invention thus provides for the genetic alteration of cells as a method to transmit therapeutic or diagnostic agents to localized regions of the blood vessel for local or systemic purposes. The range of recombinant proteins which may be expressed in these cells is broad and varied. It includes gene transfer using vectors expressing such proteins

as tPA for the treatment of thrombosis and restenosis, angiogenesis or growth factors for the purpose of revascularization, and vasoactive factors to alleviate vasoconstriction or vasospasm. This technique can also be extended to genetic treatment of inherited disorders, or acquired diseases, localized or systemic. The present invention may also be used to introduce normal cells to specific sites of cell loss, for example, to replace endothelium damaged during angioplasty or catheterization.

For example, in the treatment of ischemic diseases (thrombotic diseases), genetic material coding for tPA or modifications thereof, urokinase or streptokinase is used to transform the cells. In the treatment of ischemic organ (e.g., heart, kidney, bowel, liver, etc.) failure, genetic material coding for recollateralization agents, such as transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), angiogenin, tumor necrosis factor α, tumor necrosis factor β, acidic fibroblast growth factor or basic fibroblast growth factor can be used. In the treatment of vasomotor diseases, genetic material coding for vasodilators or vasoconstrictors may be used. These include atrial natriuretic factor, platelet-derived growth factor or endothelin. In the treatment of diabetes, genetic material coding for insulin may be used.

The present invention can also be used in the treatment of malignancies by placing the transformed cells in proximity to the malignancy. In this application, genetic material coding for diphtheria toxin, pertussis toxin, or cholera toxin may be used.

In the use of the present invention in the treatment of AIDS, genetic material coding for soluble CD4 or derivatives

thereof may be used. In the treatment of genetic diseases, for example, growth hormone deficiency, genetic material coding for the needed substance, for example, human growth hormone, is used. All of these genetic materials are readily available to one skilled in this art.

In another embodiment, the present invention provides a kit for treating a disease in a patient which contains a catheter and a solution which contains either an enzyme or a mild detergent, in which the catheter is adapted for insertion into a blood vessel and contains a main catheter body having a balloon element adapted to be inserted into said vessel and expansible against the walls of the blood vessel so as to hold the main catheter body in place in the blood vessel, and means carried by the main catheter body for delivering a solution into the blood vessel, and the solution which contains the enzyme or mild detergent is a physiologically acceptable solution. The solution may contain a proteolytic enzyme, such as dispase, trypsin, collagenase, papain, pepsin, or chymotrypsin. In addition to proteolytic enzymes, lipases may be used. As a mild detergent, the solution may contain NP-40, Triton X100, deoxycholate, SDS or the like.

Alternatively, the kit may contain a physiological acceptable solution which contains an agent such as heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material, or bivalent antibodies. This solution may also contain vectors or cells (normal or transformed). In yet another embodiment the kit may contain a catheter and both a solution which contains an enzyme or mild detergent and a solution which contains an agent such as heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material or bivalent antibody and which may optionally contain

vectors or cells.

The kit may contain a catheter with a single balloon and central distal perfusion port, together with acceptable solutions to allow introduction of cells in a specific organ or vectors into a capillary bed or cells in a specific organ or tissue perfused by this capillary bed.

Alternatively, the kit may contain a main catheter body which has two spaced balloon elements adapted to be inserted in a blood vessel with both being expansible against the walls of the blood vessel for providing a chamber in the blood vessel, and to hold the main catheter body in place. In this case, the means for delivering a solution into the chamber is situated in between the balloon elements. The kit may contain a catheter which possesses a plurality of port means for delivering the solution into the blood vessel.

Thus, the present invention represents a method for treating a disease in a patient by causing a cell attached onto the walls of a vessel or the cells of an organ perfused by this vessel in the patient to express an exogeneous therapeutic agent protein, wherein the protein treats the disease or may be useful for diagnostic purposes. The present method may be used to treat diseases, such as an ischemic disease, a vasomotor disease, diabetes, a malignancy, AIDS or a genetic disease.

The present method may use exogeneous therapeutic agent proteins, such as TPA and modifications thereof, urokinase, streptokinase, acidic fibroblast growth factor, basic fibroblast growth factor, tumor necrosis factor α, tumor necrosis factor β, transforming growth factor α, transforming growth factor β, atrial natriuretic factor, platelet-derived

growth factor, endothelian, insulin, diphtheria toxin, pertussis toxin, cholera toxin, soluble CD4 and derivatives thereof, and growth hormone to treat diseases.

The present method may also use exogenous proteins of diagnostic value. For example, a marker protein, such as β-galatosodase, may be used to monitor cell migration.

It is preferred, that the cells caused to express the exogenous therapeutic agent protein be endothelial cells.

Other features of the present invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

The data reported below demonstrate the feasibility of endothelial cell transfer and gene transplantation; that endothelial cells may be stably implanted in situ on the arterial wall by catheterization and express a recombinant marker protein, β-galactosidase, in vivo.

Because atherogenesis in swine has similarities to humans, an inbred pig strain, the Yucatan minipig (Charles River Laboratories, Inc., Wilmington, MA), was chosen as an animal model (1). A primary endothelial cell line was established from the internal jugular vein of an 8 month-old female minipig. The endothelial cell identity of this line was confirmed in that the cells exhibited growth characteristics and morphology typical of-porcine endothelium in tissue culture. Endothelial cells also express receptors for the acetylated form of low density lipoprotein (AcLDL), in contrast to fibroblasts and other mesenchymal cells (2). When analyzed

for AcLDL receptor expression, greater than 99% of the cultured cells contained this receptor, as judged by fluorescent AcLDL uptake.

Two independent β-galactosidase-expressing endothelial lines were isolated following infection with a murine amphotropic β-galactosidase-transducing retroviral vector (BAG), which is replication-defective and contains both β-galactosidase and neomycin resistance genes (3). Cells containing this vector were selected for their ability to grow in the presence of G-418. Greater than 90% of selected cells synthesized β-galactosidase by histochemical staining. The endothelial nature of these genetically altered cells was also confirmed by analysis of fluorescent AcLDL uptake. Infection by BAG retrovirus was further verified by Southern blot analysis which revealed the presence of intact proviral DNA at approximately one copy per genome.

Endothelial cells derived from this inbred strain, being syngeneic, were applicable for study in more than one minipig, and were tested in nine different experimental subjects. Under general anesthesia, the femoral and iliac arteries were exposed, and a catheter was introduced into the vessel (Figure 1). Intimal tissues of the arterial wall were denuded mechanically by forceful passage of a partially inflated balloon catheter within the vessel. The artery was rinsed with heparinized saline and incubated with the neutral protease, dispase (50 U/ml), which removed any remaining luminal endothelial cells. Residual enzyme was rapidly inactivated by α2 globulin in plasma upon deflating the catheter balloons and allowing blood to flow through the vessel segment. The cultured endothelial cells which expressed β-galactosidase were introduced using a specially designed arterial catheter (USCI,

Billerica, MA) that contained two balloons and a central instillation port (Figure 1).

When these balloons were inflated, a protected space was created within the artery into which cells were instilled through the central port 3 (Figure 1). These endothelial cells, which expressed β-galactosidase, were allowed to incubate for 30 minutes to facilitate their attachment to the denuded vessel. The catheter was then removed, the arterial branch ligated, and the incision closed.

Segments of the artery innoculated with β-galactosidase-expressing endothelium were removed 2 to 4 weeks later. Gross examination of the arterial specimen after staining using the X-gal chromagen showed multiple areas of blue coloration, compared to an artery seeded with uninfected endothelium, indicative of β-galactosidase activity. Light microscopy documented β-galactosidase staining primarily in endothelial cells of the intima in experimentally seeded vessels.

In contrast, no evidence of similar staining was observed in control segments which had received endothelial cells containing no β-galactosidase. β-Galactosidase staining was occasionally evident in deeper intimal tissues, suggesting entrapment or migration of seeded endothelium within the previously injured vessel wall. Local thrombosis was observed in the first two experimental subjects. This complication was minimized in subsequent studies by administering acetylsalicylic acid prior to the endothelial cell transfer procedure and use of heparin anticoagulation at the time of innoculation. In instances of thrombus formation, β-galactosidase staining was seen in endothelial cells

extending from the vessel wall to the surface of the thrombus.

A major concern of gene transplantation in vivo relates to the production of replication-competent retrovirus from genetically engineered cells. In these tests, this potential problem has been minimized through the use of a replication defective retrovirus. No helper virus was detectable among these lines after 20 passages in vitro. Although defective viruses were used because of their high rate of infectivity and their stable integration into the host cell genome (4), this approach to gene transfer is adaptable to other viral vectors.

A second concern involves the longevity of expression of recombinant genes in vivo. Endothelial cell expression of β-galactosidase appeared constant in vessels examined up to six weeks after introduction into the blood vessel in the present study.

These tests have demonstrated that genetically altered endothelial cells can be introduced into the vascular wall of the Yucatan minipig by arterial catheterization. Thus, the present method can be used for the localized biochemical treatment of vascular disease using genetically-altered endothelium as a vector.

A major complication of current interventions for vascular disease, such as balloon angioplasty or insertion of a graft into a diseased vessel, is disruption of the atherosclerotic plaque and thrombus formation at sites of local tissue trauma (5). In part, this is mediated by endothelial cell injury (6). The present data show that genetically-altered endothelial cells can be introduced at the time of intervention to minimize local thrombosis.

This technique can also be used in other ischemic settings, including unstable angina or myocardial infarction. For instance, antithrombotic effects can be achieved by introducing cells expressing genes for tissue plasminogen activator or urokinase. This technology is also useful for the treatment of chronic tissue ischemia. For example, elaboration of angiogenic or growth factors (7) to stimulate the formation of collateral vessels to severely ischemic tissue, such as the myocardium. Finally, somatic gene replacement for systemic inherited diseases is feasible using modifications of this endothelial cell gene transfer technique.

Experimental section:

A. Analysis of AcLDL receptor expression in normal and β-galactosidase-transduced porcine endothelial cells.

Endothelial cell cultures derived from the Yucatan minipig, two sublines infected with BAG retrovirus or 3T3 fibroblast controls were analyzed for expression of AcLDL receptor using fluorescent labelled AcLDL.

Endothelial cells were derived from external jugular veins using the neutral protease dispase (8). Excised vein segments were filled with dispase (50 U/ml in Hanks' balanced salt solution) and incubated at 30°C for 20 minutes. Endothelium obtained by this means was maintained in medium 199 (GIBCO, Grand Island, N.Y.) supplemented with fetal calf serum (10%), 50 μmg/ml endothelial cell growth supplement (ECGS) and heparin (100 μg/ml). These cells were infected with BAG retrovirus, and selected for resistance to G-418. Cell cultures were incubated with (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbacyanine perchlorate) (DiI) AcLDL (Biomedical

Technologies, Stoughton, MA) (10 μg/ml) for 4-6 hrs. at 37°C, followed by three rinses with phosphate-buffered saline containing 0.5% glutaraldehyde. Cells were visualized by phase contrast and fluorescent microscopy.

B. Method of introduction of endothelial cells by catheterization.

A double balloon catheter was used for instillation of endothelial cells. The catheter has a proximal and distal balloon, each 6 mm in length and 5 mm in width, with a 20 mm length between the balloons. The central section of the catheter has a 2 mm pore connected to an instillation port. Proximal and distal balloon inflation isolates a central space, allowing for instillation of infected cells through the port into a discrete segment of the vessel. For a schematic representation of cell introduction by catheter, see Figures 1 and 2.

Animal care was carried out in accordance with "Principles of Laboratory Animal Care" and "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, Revised 1978). Female Yucatan minipigs (80-100 kg) were anesthetized with pentobarbital (20 mg/kg), intubated, and mechanically ventilated. These subjects underwent sterile surgical exposure of the iliac and femoral arteries. The distal femoral artery was punctured, and the double-balloon catheter was advanced by guidewire into the iliac artery. The external iliac artery was identified; the proximal balloon was partially inflated and passed proximally and distally so as to mechanically denude the endothelium. The catheter was then positioned with the central space located in the region of denuded endothelium, and both balloons were inflated. The denuded segment was irrigated with

heparinized saline, and residual adherent cells were removed by instillation of dispase (20 U/ml) for 10 min. The denuded vessel was further irrigated with a heparin solution and the BAG-infected endothelial cells were instilled for 30 min. The balloon catheter was subsequently removed, and antegrade blood flow was restored. The vessel segments were excised 2 to 4 weeks later. A portion of the artery was placed in 0.5% glutaraldehyde for five minutes and stored in phosphate-buffered saline, and another portion was mounted in a paraffin block for sectioning. The presence of retroviral expressed β-galactosidase was determined by a standard histochemical technique (19).

C. Analysis of endothelial cells in vitro and in vivo.

β-Galactosidase activity was documented by histochemical staining in (A) primary endothelial cells from the Yucatan minipig, (B) a subline derived by infection with the BAG retroviral vector, (C) a segment of normal control artery, (D) a segment of artery instilled with endothelium infected with the BAG retroviral vector, (E) microscopic cross-section of normal control artery, and (F) microscopic cross-section of artery instilled with endothelium infected with the BAG retroviral vector.

Endothelial cells in tissue culture were fixed in 0.5% glutaraldehyde prior to histochemical staining. The enzymatic activity of the E. coli β-galactosidase protein was used to identify infected endothelial cells in vitro and in vivo. The β-galactosidase transducing Mo-MuLV vector (2), (BAG) was kindly provided by Dr. Constance Cepko. This vector used the wild type MoMuLV LTR as a promoter for the β-galactosidase gene. The simian virus 40 (SV-40) early promoter linked to the

Tn5 neomycin resistance gene provides resistance to the drug G-418 and is inserted downstream of the β-galactosidase gene, providing a marker to select for retrovirus-containing, β-galactosidase expressing cells. This defective retrovirus was prepared from fibroblast ψ am cells (3,10), and maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% calf serum. Cells were passaged twice weekly following trypsinization. The supernatant, with titers of 10^4-10^5/ml G-418 resistant colonies, was added to endothelial cells at two-thirds confluence and incubated for 12 hours in DMEM with 10% calf serum at 37°C in 5% CO_2 in the presence of 8 μg/ml of polybrene. Viral supernatants were removed, and cells maintained in medium 199 with 10% fetal calf serum, ECGS (50 μg/ml), and endothelial cell conditioned medium (20%) for an additional 24 to 48 hours prior to selection in G-418 (0.7 μg/ml of a 50% racemic mixture). G-418 resistant cells were isolated and analyzed for β-galactosidase expression using a standard histochemical stain (9). Cells stably expressing the β-galactosidase enzyme were maintained in continuous culture for use as needed. Frozen aliquots were stored in liquid nitrogen.

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Obviously, numerous modifications and variations of the present invention are possible in light of the above-teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

Claims:

1. A kit for treating a disease in a patient in need thereof, comprising a catheter means and a solution which contains an enzyme or mild detergent, wherein:

(i) said catheter means is adapted for insertion into a blood vessel and comprises a main catheter body having means including a balloon element adapted to be inserted into said vessel and expansible against the walls of said vessel so as to hold said main catheter body in place in said vessel, and means carried by said main catheter body for delivering a solution into said blood vessel; and

(ii) said solution is a physiologically acceptable solution.

2. The kit of Claim 1, wherein said solution contains, as said enzyme, at least one member selected from the group consisting of dispase, trypsin, collagenase, papain, pepsin, chymotrypsin, and lipases.

3. The kit of Claim 1, wherein said solution contains at least one member selected from the group consisting of NP-40, Triton X100, deoxycholate, and SDS.

4. The kit of Claim 1, wherein said main catheter body comprises means including two spaced balloon elements, adapted to be inserted in a blood vessel and both being expansible against the walls of the blood vessel, for providing a chamber in said blood vessel and so as to hold said main catheter body in place, and whereas said means for delivering a solution into said chamber is situated in between said balloon elements.

5. The kit of Claim 1, wherein said means for delivering said solution into said blood vessel comprises a plurality of pore means.

6. A kit for treating a disease in a patient in need thereof, comprising a catheter means and a physiologically acceptable solution, wherein:

(i) said catheter means is adapted for insertion into a blood vessel and comprises a main catheter body having means including a balloon element, adapted to be inserted in said blood vessel and being expansible against the walls of said vessel so as to hold said main catheter body in place, and means carried by said main catheter body for delivering a solution into said blood vessel;

(ii) said physiologically acceptable solution comprises at least one member selected from the group consisting of heparin, poly-L-lysine, polybrene, dextransulfate, a polycationic material, and bivalent antibodies.

7. The kit of Claim 6, wherein said physiologically acceptable solution further comprises DNA.

8. The kit of Claim 6, wherein said physiologically acceptable solution further comprises a growth factor.

9. A method for treating a disease in a patient in need thereof, comprising causing a cell attached onto the walls of a vessel or in an organ or tissue in said patient to express an exogeneous therapeutic agent protein, wherein said protein treats said disease.

10. The method of Claim 9, wherein said disease is an ischemic disease, a vasomotor disease, diabetes, a malignancy, AIDS or a genetic disease.

11. The method of Claim 9, wherein said disease is a systemic disease.

12. The method of Claim 9, wherein said exogeneous therapeutic agent protein is one member selected from the group consisting of tPA and modifications thereof, urokinase, streptokinase, acidic fibroblast growth factor, basic fibroblast growth factor, tumor necrosis factor α, tumor necrosis factor β, transforming growth factor α, transforming growth factor β, atrial natriuretic factor, platelet-derived growth factor, endothelian, insulin, diphtheria toxin, pertussis toxin, cholera toxin, soluble CD4 and derivatives thereof, and growth hormone.

13. The method of Claim 9, wherein said cell is selected from the group consisting of endothelial cells, vascular smooth muscle cells, fibroblasts, connective tissue cells, macrophages, monocytes, and parenchymal cells.

14. A method for treating a disease, comprising site-specifically instilling cells.

15. The method of Claim 14, wherein said cells are transformed cells.

16. The method of Claim 14, wherein said cells are normal cells.

17. A method for treating a disease, comprising site-specifically transforming cells in vivo.

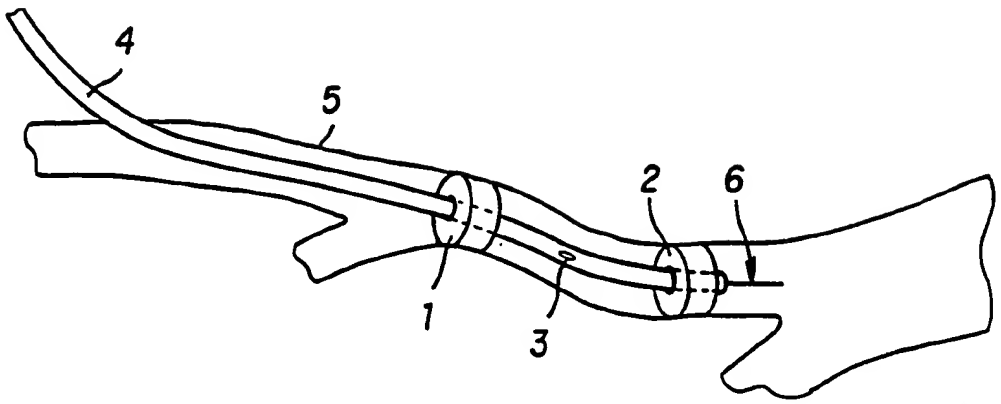

FIG. 1

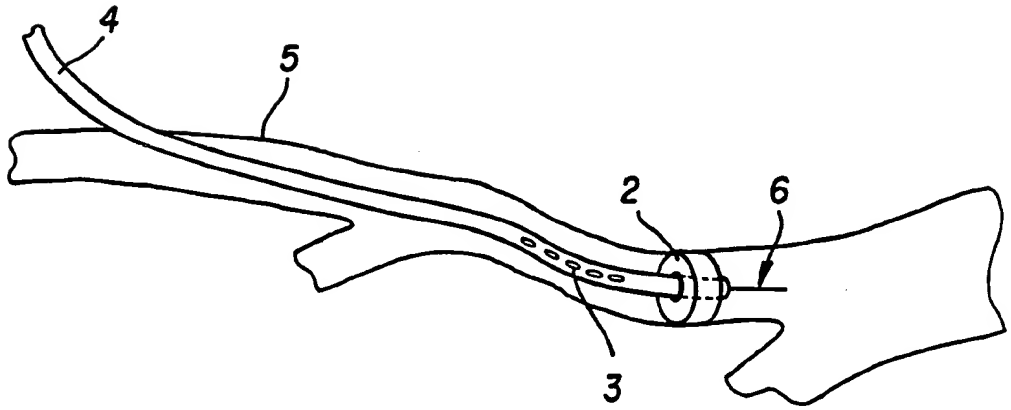

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01662

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61F 2/06; A61M 29/02; A61K 37/54,37/02 39/10,39/106,31/70

US CL.: 623/11; 604/101; 424/94.6, 94.63, 93, 92; 514/2, 21, 56, 12, 44

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	623/11; 604/101; 424/94.6, 94.63, 94.64, 92, 93; 514/2, 12, 21, 44, 56; 435/240.22

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,Y	US,A, 4,874,746 (ANTONIADES, ET AL) issued 17 October 1989, See the entire document	17
P,X/Y	US,A, 4,824,436 (WOLINSKY) issued 25 April 1989 See the entire document	6/7,8
X	US,A, 4,636,195 (WOLINSKY) issued 13 January 1987 See the entire document	1 - 5
X	US,A, 4,353,888 (SEFTON) issued 12 October 1982 See the entire document	9-14, 16
X	US,A, 4,332,893 (ROSENBERG) issued 01 June 1982 See the entire document	9-12, 14-16
X/Y	(ZWIEBEL), "High-Level Recombinant Gene Expression in Rabbit Endothelial Cells Transduced by Retroviral Vectors", Science, issued January 1989, Volume 243, pages 220-222, see the entire document	9-15, 6-8
P,X	(NABEL) "Recombinant Gene Expression in vivo Within Endothelial Cells of the Arterial Wall", Science, issued June 1989, Volume 244, pages 1342-1344, see the entire document	1 - 16

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
13 June 1990	26 JUL 1990
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	J.M. Stone

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	(SELDEN), "Implantation of Genetically Engineered Fibroblasts into Mice: Implications for Gene Therapy", Science, issued M?ay 1987, Volume 236, pages 714-718, see the entire document	9-15

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.